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# Antihyperalgesia by $\alpha 2$ -GABA<sub>A</sub> Receptors Occurs Via a Genuine Spinal Action and Does Not Involve Supraspinal Sites

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Drugs that enhance GABAergic inhibition alleviate inflammatory and neuropathic pain after spinal application. This antihyperalgesia occurs mainly through GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) containing  $\alpha 2$  subunits ( $\alpha 2$ -GABA<sub>A</sub>Rs). Previous work indicates that potentiation of these receptors in the spinal cord evokes profound antihyperalgesia also after systemic administration, but possible synergistic or antagonistic actions of supraspinal  $\alpha 2$ -GABA<sub>A</sub>Rs on spinal antihyperalgesia have not yet been addressed. Here we generated two lines of GABA<sub>A</sub>R-mutated mice, which either lack  $\alpha 2$ -GABA<sub>A</sub>Rs specifically from the spinal cord, or, which express only benzodiazepine-insensitive  $\alpha 2$ -GABA<sub>A</sub>Rs at this site. We analyzed the consequences of these mutations for antihyperalgesia evoked by systemic treatment with the novel non-sedative benzodiazepine site agonist HZ166 in neuropathic and inflammatory pain. Wild-type mice and both types of mutated mice had similar baseline nociceptive sensitivities and developed similar hyperalgesia. However, antihyperalgesia by systemic HZ166 was reduced in both mutated mouse lines by about 60% and was virtually indistinguishable from that of global point-mutated mice, in which all  $\alpha 2$ -GABA<sub>A</sub>Rs were benzodiazepine insensitive. The major ( $\alpha 2$ -dependent) component of GABA<sub>A</sub>R-mediated antihyperalgesia was therefore exclusively of spinal origin, whereas supraspinal  $\alpha 2$ -GABA<sub>A</sub>Rs had neither synergistic nor antagonistic effects on antihyperalgesia. Our results thus indicate that drugs that specifically target  $\alpha 2$ -GABA<sub>A</sub>Rs exert their antihyperalgesic effect through enhanced spinal nociceptive control. Such drugs may therefore be well-suited for the systemic treatment of different chronic pain conditions.

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**Keywords:** neuropathic pain; analgesia; GABA; benzodiazepine; spinal cord; mouse model

## INTRODUCTION

Chronic neuropathic pain syndromes are frequently unresponsive to classical analgesic drugs including cyclooxygenase inhibitors and opioids. Drugs most effective in these pain conditions include anticonvulsant drugs that modulate or block voltage-gated Na<sup>+</sup> or Ca<sup>2+</sup> channels (Sang and Hayes, 2006). Other anticonvulsive drugs with a different mode of action include the benzodiazepine site agonists, which enhance neuronal inhibition through facilitation of GABA<sub>A</sub> receptor-mediated neurotransmission. Diminished GABAergic or glycinergic inhibition in the spinal dorsal horn (ie, in the sensory part of the spinal cord)

has been shown to be a major contributor to chronic pain syndromes (Ahmadi *et al*, 2002; Coull *et al*, 2003; Harvey *et al*, 2004), suggesting that drugs that facilitate spinal inhibition might correct a major component of the maladaptive neuroplasticity underlying chronic pain states. In line with this concept, previous work has shown that spinal injection of benzodiazepine site agonists provides pain relief in a number of rodent models of inflammatory and neuropathic pain (Knabl *et al*, 2008; Luger *et al*, 1995; Witschi *et al*, 2011).

Mammalian GABA<sub>A</sub>Rs form a heterogeneous family of heteropentameric ion channels assembled from a repertoire of 19 subunits. The most prevalent subtypes of GABA<sub>A</sub>Rs contain two  $\alpha$ -, two  $\beta$ - and one  $\gamma 2$ - subunits. Pharmacological properties of the different GABA<sub>A</sub>R subtypes are best characterized by the type of  $\alpha$ -subunit present in the individual receptors (Olsen and Sieghart, 2008). Experiments in genetically modified mice demonstrated a particular relevance of GABA<sub>A</sub>Rs with an  $\alpha 2$ -type benzodiazepine pharmacology ( $\alpha 2$ -GABA<sub>A</sub>Rs) for antihyperalgesia mediated

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by spinally applied benzodiazepines (Knabl *et al*, 2008). These and subsequent experiments (Knabl *et al*, 2009) established that the antihyperalgesic actions of benzodiazepine site agonists occur independently from the sedative action, which is mediated by  $\alpha 1$ -GABA<sub>A</sub>Rs (Rudolph *et al*, 1999). More recent experiments using novel benzodiazepine site ligands with improved subunit specificity (ie, reduced or absent activity at  $\alpha 1$ -GABA<sub>A</sub>Rs) have shown that such novel compounds reduce nerve injury-induced and inflammation-induced hyperalgesia also after systemic administration (Di Lio *et al*, 2011; Knabl *et al*, 2008; Knabl *et al*, 2009; Munro *et al*, 2009; Munro *et al*, 2008; Reichl *et al*, 2012). While the contribution of spinal GABA<sub>A</sub>Rs to this antihyperalgesia is likely, although still not formally proven, the relevance of GABA<sub>A</sub>R subtypes in supraspinal circuits is unclear. Such supraspinal GABA<sub>A</sub>Rs might contribute to antihyperalgesia through a genuine antihyperalgesic effect, eg, through GABA<sub>A</sub>Rs in the rostral agranular insular cortex (Jasmin *et al*, 2003), or indirectly through the reversal of anxiety-induced or stress-induced hyperalgesia (Andre *et al*, 2005). The latter possibility is particularly relevant as  $\alpha 2$ -GABA<sub>A</sub>Rs also contribute to benzodiazepine-mediated anxiolysis (Löw *et al*, 2000; Morris *et al*, 2006). In contrast, supraspinal GABA<sub>A</sub>Rs might also counteract spinal antihyperalgesia through the silencing antinociceptive tracts descending from the periaqueductal gray or the rostroventromedial medulla (Harris and Westbrook, 1995; Luger *et al*, 1995; Tatsuo *et al*, 1999). It is thus possible that activation of supraspinal GABA<sub>A</sub>Rs either facilitates or constrains spinal antihyperalgesia.

To address these questions, we have generated two lines of GABA<sub>A</sub>R-mutated mice. The first line (*hoxb8- $\alpha 2$ <sup>-/-</sup>*) carries a tissue-specific deletion of the GABA<sub>A</sub>R  $\alpha 2$  subunit from all spinal neurons, astrocytes, and primary sensory neurons up to the mid cervical level (approximately C4). This tissue-specific ablation was achieved by crossing mice that carried a GABA<sub>A</sub>R  $\alpha 2$  (*Gabra2*) allele flanked by two loxP sites ( $\alpha 2^{\text{fl}}$ ; Witschi *et al*, 2011) with mice expressing the cre recombinase under the transcriptional control of the *hoxb8* homeobox gene (Witschi *et al*, 2010). The second line can be viewed as a tissue-specific point-mutated  $\alpha 2$ -GABA<sub>A</sub>R mouse line (*hoxb8- $\alpha 2$ <sup>R/-</sup>*), which carries in addition to one  $\alpha 2^{\text{fl}}$  allele, a benzodiazepine-insensitive H101R point-mutated *Gabra2* allele ( $\alpha 2^{\text{R}}$ ; Löw *et al*, 2000), and the *hoxb8*-cre transgene. At supraspinal sites, this line expresses the point-mutated allele together with a fully functional ('wild-type')  $\alpha 2^{\text{fl}}$  allele, whereas in primary sensory neurons and in the spinal nervous system only the point-mutated allele is expressed. For pharmacological analyses, we used the novel non-sedative 8-acetyleno-2'-pyridoimidazobenzodiazepine HZ166 (ethyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, compound 2 in Rivas *et al*, 2009), which has previously been shown to exhibit antihyperalgesic properties in the absence of sedation in mice (Di Lio *et al*, 2011). Analysis of the antihyperalgesic effects of HZ166 in the two mutated mouse lines and comparison of these effects with those obtained in wild-type mice and in mice in which all  $\alpha 2$ -GABA<sub>A</sub>Rs had been rendered benzodiazepine-insensitive revealed that activation of supraspinal  $\alpha 2$ -GABA<sub>A</sub>Rs neither exerts a positive nor a negative impact on the antihyperalgesic actions of systemically applied HZ166.

## MATERIALS AND METHODS

### Generation of GABA<sub>A</sub>R-Mutated Mice

Three strains of GABA<sub>A</sub>R  $\alpha 2$  (*gabra2*)-mutated mice were used to generate the genotypes needed for the present study: (i) mice carrying GABA<sub>A</sub>R  $\alpha 2$  alleles flanked by loxP sites ( $\alpha 2^{\text{fl}}$ ; Witschi *et al*, 2011), (ii) mice carrying a H101R point-mutated 'knock-in' allele ( $\alpha 2^{\text{R}}$ ; Löw *et al*, 2000), and (iii) *hoxb8*-cre transgenic mice (Witschi *et al*, 2010). *Hoxb8- $\alpha 2$ <sup>-/-</sup>* mice and *hoxb8*-cre-negative ( $\alpha 2^{\text{fl/fl}}$ ) littermates were obtained from crossings of *hoxb8*-cre-positive  $\alpha 2^{\text{fl/fl}}$  mice with  $\alpha 2^{\text{fl/fl}}$  mice. *Hoxb8- $\alpha 2$ <sup>R/-</sup>* mice and *hoxb8*-cre-negative ( $\alpha 2^{\text{R/fl}}$ ) littermates were generated by crossing *hoxb8*-cre-positive  $\alpha 2^{\text{fl/fl}}$  mice with  $\alpha 2^{\text{R/R}}$ . All mouse lines had been backcrossed to a C57BL/6J background for at least 10 generations and were maintained on this background throughout the study. During the breeding of *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice, a small number of mice were born carrying a non-conditional knockout allele of the *gabra2* gene, which had occurred through undesired recombination events in the germline. To ensure that these mice were excluded from all experiments and breeding, we verified the absence of the knockout allele through PCR analyses performed on DNA extracted from ear biopsies. This tissue was chosen because analysis of *hoxb8*-cre ROSA26-lacZ double transgenic reporter mice had demonstrated that *hoxb8*-cre was not expressed in outer ear tissue at any developmental stage.

### Morphology

Distribution of GABA<sub>A</sub>R  $\alpha 2$  protein was studied in brain and spinal cord sections obtained from adult *hoxb8- $\alpha 2$ <sup>-/-</sup>* and *hoxb8- $\alpha 2$ <sup>R/-</sup>* mice and from *hoxb8*-cre<sup>-</sup> littermates ( $\alpha 2^{\text{fl/fl}}$  and  $\alpha 2^{\text{R/fl}}$  mice). For immunoperoxidase stainings, a polyclonal antibody directed against the N-terminal nine amino acids (NIQEDEAKN) of the GABA<sub>A</sub>R  $\alpha 2$  subunit (Fritschy and Möhler, 1995) was used as the primary antibody. Stainings (triplicates) were made as described previously (Paul *et al*, 2012). Sections from GABA<sub>A</sub> receptor-mutated mice and from control littermates were treated in a strictly parallel fashion.

### Quantitative reverse Transcriptase PCR

Four to six lumbar spinal cords, lumbar DRGs, and hippocampi were rapidly removed from euthanized adult *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice and  $\alpha 2^{\text{fl/fl}}$  littermates. mRNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen no.205311). Expression of GABA<sub>A</sub>R subunits was assessed using  $\beta$ -actin as reference gene (for details of the assays see Witschi *et al* (2011)).

### [<sup>3</sup>H]Ro 15-4513 Binding Assay

HEK293 cells maintained in DMEM/10% FBS in polylysine-coated culture dishes (10 cm) were transfected with plasmids containing the subunit combination  $\alpha 2\beta 3\gamma 2$  or  $\alpha 2(\text{H101R})\beta 3\gamma 2$  (7 mg total DNA, ratio 1:1:2) using jetPEI transfection reagent (Polyplus-transfection). Twenty-four hours after transfection, HEK293 cells were collected in PBS. HEK293 cells were homogenized in 10 vol 10 mM Tris pH 7.5, 0.32 M sucrose, protease inhibitor cocktail (complete

Mini, Roche Applied Science) and centrifuged at 1000 g for 10 min. The supernatant was carefully removed and centrifuged at 4 °C again for 20 min at 25 000 g at 4 °C. The crude membrane pellet was resuspended in 10 mM Tris-HCl pH 7.4, protease inhibitor cocktail and washed once by centrifugation and re-suspension. Aliquots of the crude membranes prepared from HEK293 cells expressing the  $\alpha 2\beta 3\gamma 2$  or  $\alpha 2(\text{H101R})\beta 3\gamma 2$  subunit combination (150–200  $\mu\text{g}$  protein) were incubated with increasing concentrations of HZ166 ( $10^{-8}$ – $10^{-4}$  M) and 6.3 nM [ $^3\text{H}$ ]Ro 15-4513 (22.7 Ci/mmol, PerkinElmer) in a total volume of 200  $\mu\text{l}$  for 90 min on ice. Subsequently, the samples were filtered onto glass fiber filters using a 12-channel semiautomated cell harvester (Scatron) and washed with ice-cold buffer (10 mM Tris-HCl pH 7.4). Non-specific [ $^3\text{H}$ ]Ro 15-4513 binding was measured using 10  $\mu\text{M}$  flumazenil. The radioactivity of the filters was determined by liquid scintillation counting using a Tricarb 2500 liquid scintillation analyzer. Binding data were analyzed using the GraphPad Prism software (version 5.02, GraphPad Software, USA).

### Electrophysiological Analyses

The effects of HZ166 on GABA<sub>A</sub>Rs were studied in HEK293 cells transiently expressing GABA<sub>A</sub>Rs and in spinal cord slices of *hoxb8- $\alpha 2^{-/-}$*  mice and wild-type (*hoxb8-cre-negative  $\alpha 2^{\text{fl/fl}}$* ) littermates. HEK293 cells were transfected with rat  $\alpha 2/\alpha 2(\text{H101R})$ ,  $\beta 3$  and  $\gamma 2$  GABA<sub>A</sub>R expression vectors (Benson *et al*, 1998) using lipofectamine LTX (Invitrogen). The transfection mixture contained (in  $\mu\text{g}$ ) 1  $\alpha 2/\beta 3$ , 3  $\gamma 2$  and 0.5 EGFP (used as a marker of successful transfection). Recordings were made 18–36 hours after transfection. Whole-cell patch-clamp recordings of GABA-evoked currents were made at room temperature (20–24 °C) and at a holding potential of  $-60$  mV. The external solution contained (in mM) 150 NaCl, 10 KCl, 2.0  $\text{CaCl}_2$ , 1.0  $\text{MgCl}_2$ , 10 HEPES (pH 7.4), and 10 glucose. Recording electrodes were filled with internal solution containing (in mM) 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4  $\text{MgCl}_2$ , 0.5 GTP, and 2 ATP. CsCl was used instead of KCl to block GABA<sub>B</sub> receptor-evoked  $\text{K}^+$  currents. GABA was applied to the recorded cell using a manually controlled pulse (4–6 s) of a low subsaturating GABA concentration ( $\text{EC}_{50}$ ). GABA  $\text{EC}_{50}$  values were determined for wild-type  $\alpha 2\beta 3\gamma 2$  and mutant  $\alpha 2(\text{H101R})\beta 3\gamma 2$  receptors separately.  $\text{EC}_{50}$  values and Hill coefficients ( $n_h$ ) were obtained from fits of normalized concentration–response curves to the equation  $I_{\text{GABA}} = I_{\text{max}} [\text{GABA}]n_h / ([\text{GABA}]n_h + [\text{EC}_{50}]n_h)$ .  $I_{\text{max}}$  was determined as the current elicited by a saturating concentration of GABA (500  $\mu\text{M}$ ). HZ166 was co-applied together with GABA without preincubation.

Transverse spinal cord slices were prepared from 2- to 3-week-old mice of either sex as described previously (Ahmadi *et al*, 2002). Slices were kept in oxygenated (95%  $\text{O}_2/5\%$   $\text{CO}_2$ ) external solution containing (in mM) 120 NaCl, 5 HEPES, 26  $\text{NaHCO}_3$ , 1.25  $\text{NaH}_2\text{PO}_4$ , 2.5 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , and 10 glucose (pH 7.35) at 35 °C. Superficial dorsal horn neurons were visually identified using an infrared gradient contrast equipment. Whole-cell patch-clamp recordings were performed at room temperature at a holding potential of  $-60$  mV. Patch pipettes were filled with

internal solution containing (in mM) 120 CsCl, 10 EGTA, 4  $\text{MgCl}_2$ , 0.5 GTP, 2 ATP, and 10 HEPES (pH 7.30 adjusted with CsOH). QX-314 (5 mM) was added to block voltage-activated  $\text{Na}^+$  currents in the recorded cell. Slices were continuously superfused with oxygenated external solution at a flow rate of 1.3–1.6 ml/min. After 4–5 min of baseline recording, GABA (50  $\mu\text{M}$ ) was bath-applied. Steady-state GABA-evoked currents were achieved usually 3–5 min after application. Subsequently, HZ166 (10  $\mu\text{M}$ ) was co-applied with the same GABA concentration for 6 to 7 min. Afterward, GABA and HZ166 were washed-out, or bicuculline (20  $\mu\text{M}$ ) was applied. Recordings in which recovery to baseline currents before GABA application was less than 85–90% were excluded from the analysis.

### Behavioral Testing

Experiments were performed in 7- to 10-week-old male and female mice. Care was taken to ensure equal numbers of age-matched male and female mice in all experiments. Mechanical and thermal nociceptive sensitivities of *hoxb8- $\alpha 2^{-/-}$*  and of *hoxb8-cre-negative  $\alpha 2^{\text{fl/fl}}$*  littermates were determined using electronic von Frey filaments and the plantar test, respectively (for details see Witschi *et al*, 2011).

Antihyperalgesic properties of HZ166 were studied in two models: (i) activity against neuropathic hyperalgesia was assessed in the chronic constriction injury (CCI) model (Bennett and Xie, 1988); unilateral constriction of the left sciatic nerve was performed as described previously (Hösl *et al*, 2006) and (ii) inflammation was evoked through subcutaneous injection of zymosan A (0.06 mg in 20  $\mu\text{l}$  saline (0.9% NaCl)) into one hindpaw. HZ166 was tested 7 days after CCI surgery or 48 h after zymosan A injection, when sensitization had reached a maximum (Witschi *et al*, 2011). Sensitivities of the injured/inflamed paw and of the contralateral control paw were measured alternately and at least five measurements were taken per mouse and time point. Antihyperalgesia was quantified for the time interval of 60–90 min post drug injection, when the drug effect was maximal, and expressed as percent maximum possible analgesia =  $(R_{\text{post-drug}} - R_{\text{pre-drug}}) / (R_{\text{baseline}} - R_{\text{pre-drug}}) \times 100\%$ , where R is the average response latency or threshold under baseline condition ( $R_{\text{baseline}}$ ), after induction of neuropathy or inflammation but before drug injection ( $R_{\text{pre-drug}}$ ), and 60–90 min after drug injection ( $R_{\text{post-drug}}$ ).

Locomotor activity was assessed as described before (Di Lio *et al*, 2011). HZ166 was administered immediately before placing the mice into the open field arena, and the number of beam crosses per 24 min was determined for a total of 96 min after drug administration.

Motor coordination of *hoxb8- $\alpha 2^{-/-}$*  and of *hoxb8-cre-negative  $\alpha 2^{\text{fl/fl}}$*  littermates was investigated in the rotarod test either at two fixed rotational speeds (5 and 10 r.p.m.) for analysis of baseline motor coordination, or with increasing rotational velocity (from 4 r.p.m. to 40 r.p.m. within 5 min) for the analysis of effects of HZ166 on motor coordination. Each mouse was tested three times. HZ166 was given 60 min before testing on the rotarod. Permission for the animal experiments was obtained from the Veterinäramt des Kantons Zürich (ref. no. 135/2009).



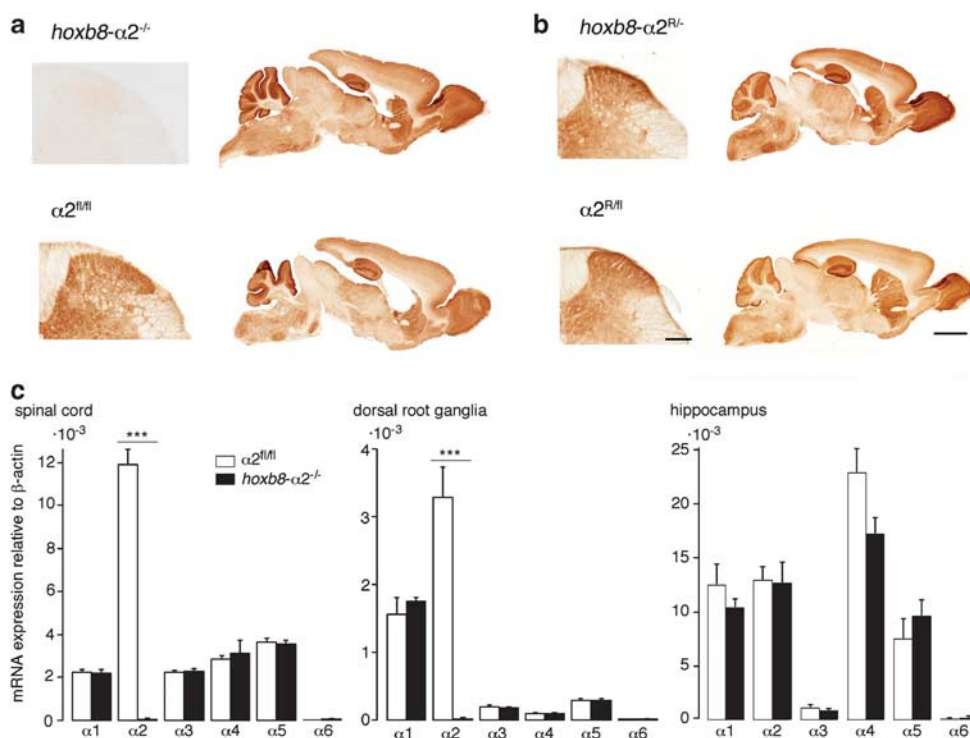
## RESULTS

To assess a possible influence of supraspinal  $\alpha 2$ -GABA<sub>A</sub>R to GABAergic analgesia, three prerequisites were needed: (i) mice that express benzodiazepine-sensitive  $\alpha 2$ -GABA<sub>A</sub>R only in the brain, (ii) a benzodiazepine site agonist that causes significant antihyperalgesia after systemic administration at doses that do not produce confounding sedation or motor impairment, and (iii) we needed to demonstrate that the facilitating action of this agonist on  $\alpha 2$ -GABA<sub>A</sub>R was lost in H101R point-mutated receptors.

In order to reach the first prerequisite, we generated two lines of  $\alpha 2$ -GABA<sub>A</sub>R-mutated mice using the cre/loxP system. The first line lacked the GABA<sub>A</sub>R  $\alpha 2$  subunit from the spinal cord and from all primary sensory neurons (*hoxb8- $\alpha 2$ <sup>-/-</sup>*), but showed unchanged GABA<sub>A</sub>R  $\alpha 2$  subunit expression in the brain. These mice carried a *hoxb8-cre* transgene and two floxed GABA<sub>A</sub>R  $\alpha 2$  ( $\alpha 2^{\text{fl}}$ ) alleles. The second line was a conditional point-mutated (*hoxb8- $\alpha 2$ <sup>R/-</sup>*) mouse line, whose spinal  $\alpha 2$ -GABA<sub>A</sub>R were rendered benzodiazepine insensitive. These mice carried the *hoxb8-cre* transgene together with a  $\alpha 2^{\text{fl}}$  allele and a point-mutated (H101R) GABA<sub>A</sub>R  $\alpha 2$  ( $\alpha 2^{\text{R}}$ ) allele. Morphological analyses demonstrated that the supraspinal GABA<sub>A</sub>R  $\alpha 2$  subunit distribution of *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice was indistinguishable from that of  $\alpha 2^{\text{fl/fl}}$  (wild-type) littermates (Figure 1a). However, transverse sections of the lower lumbar spinal

cord of *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice did not show any GABA<sub>A</sub>R  $\alpha 2$  subunit immunoreactivity, indicating highly effective *hoxb8-cre*-mediated gene recombination. Sagittal sections of the cervical spinal cord revealed the expected progressive rostral to caudal loss of GABA<sub>A</sub>R  $\alpha 2$  subunit expression within the upper cervical segments (not shown). The apparent lack of  $\alpha 2$ -GABA<sub>A</sub>R from the lumbar spinal cord of *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice indicates that only few, if any, spinal  $\alpha 2$ -GABA<sub>A</sub>R reside on processes of neurons descending from supraspinal areas. In conditional point-mutated (*hoxb8- $\alpha 2$ <sup>R/-</sup>*) mice and *hoxb8-cre*-negative ( $\alpha 2^{\text{R/fl}}$ ) littermates, no differences in  $\alpha 2$ -subunit immunoreactivity were observed in either the brain or the spinal cord (Figure 1b), suggesting that the loss of one allele had no apparent effect on the amount of GABA<sub>A</sub>R  $\alpha 2$  subunit expressed.

To further corroborate the loss of GABA<sub>A</sub>R  $\alpha 2$  gene expression and to assess possible compensatory changes in the expression of other GABA<sub>A</sub>R  $\alpha$ -subunits, we performed qRT-PCR analyses from lumbar DRGs, lumbar spinal cords, and hippocampi of *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice and *hoxb8-cre*-negative  $\alpha 2^{\text{fl/fl}}$  (wild-type) littermates (Figure 1c). In all three tissues, we detected mRNA encoding for GABA<sub>A</sub>R  $\alpha 1$ – $\alpha 5$  subunits, but no  $\alpha 6$  subunit mRNA. As expected, the  $\alpha 2$  subunit was the most extensively expressed GABA<sub>A</sub>R  $\alpha$ -subunit in the lumbar spinal cords and DRGs. Its expression was completely lost in lumbar DRGs and spinal



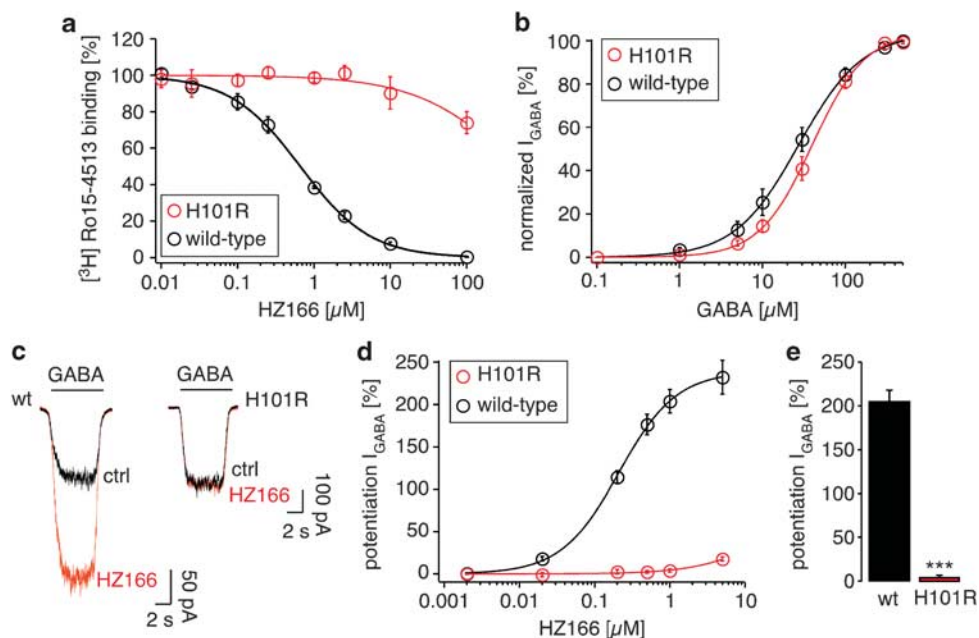
**Figure 1** GABA<sub>A</sub>R  $\alpha 2$ -subunit distribution in brains and spinal cords of *hoxb8- $\alpha 2$ <sup>-/-</sup>* and *hoxb8- $\alpha 2$ <sup>R/fl</sup>* mice and changes in GABA<sub>A</sub>R  $\alpha$ -subunit expression. (a, b), immunoperoxidase staining of GABA<sub>A</sub>R  $\alpha 2$  subunits in horizontal sections of the lumbar spinal dorsal horn and in sagittal brain sections of spinal cord-specific GABA<sub>A</sub>R  $\alpha 2$ -knockout (*hoxb8- $\alpha 2$ <sup>-/-</sup>*) and  $\alpha 2$ (H101R)-knock-in mice (*hoxb8- $\alpha 2$ <sup>R/-</sup>*). Bottom row, sections obtained from the respective control littermates (*hoxb8-cre*-negative wild-type and  $\alpha 2^{\text{R/fl}}$ ). Similar results were obtained from at least two additional mice of each genotype. Scale bars are 200  $\mu$ m (dorsal horn) and 1 mm (brain), and apply to all sections. (c) Quantitative reverse transcriptase PCR measurements of all six GABA<sub>A</sub>R  $\alpha$ -subunits in lumbar DRGs, lumbar spinal cords, and hippocampi of *hoxb8- $\alpha 2$ <sup>-/-</sup>* and  $\alpha 2^{\text{fl/fl}}$  littermates. mRNA expression relative to  $\beta$ -actin (mean  $\pm$  SEM,  $n = 6$ –7 mice per genotype and tissue). mRNA encoding for  $\alpha 6$  GABA<sub>A</sub>R subunit was not detectable in any of the three tissues. \*\*\* $P < 0.001$ ,  $P > 0.13$  for all other comparisons, ANOVA followed by Bonferroni *post hoc* correction for 18 independent pair-wise comparisons.

cords of *hoxb8- $\alpha 2^{-/-}$*  mice, but remained unchanged in the hippocampus. No significant changes were seen in the expression of the other GABA<sub>A</sub>  $\alpha$  subunits in any of the three tissues. For spinal cord and DRG tissue, we also analyzed the expression of  $\rho 1$ ,  $\rho 2$ , and  $\rho 3$  subunit genes, which are able to form functional, benzodiazepine-insensitive GABA<sub>A</sub> (or sometimes also called GABA<sub>C</sub>) receptors. Expression of  $\rho$ -subunits was unchanged in *hoxb8- $\alpha 2^{-/-}$*  mice (data not shown).

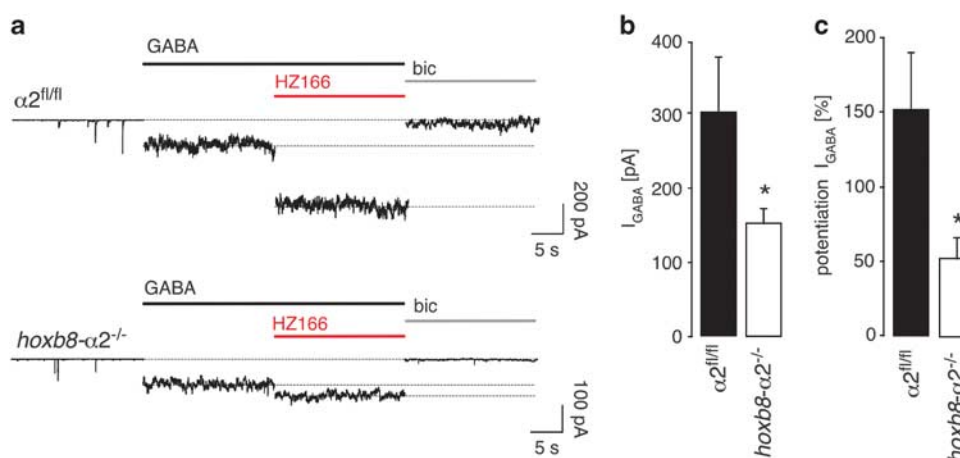
In order to assess the contribution of supraspinal  $\alpha 2$ -GABA<sub>A</sub>Rs to benzodiazepine-mediated antihyperalgesia, we used the new non-sedative partial benzodiazepine site agonist HZ166 (Rivas et al, 2009), which exerts pronounced antihyperalgesic actions in mice in the absence of sedation (Di Lio et al, 2011). Among the different available compounds with improved subtype specificity, we chose HZ166 because it possesses higher intrinsic activity at  $\alpha 2$ -GABA<sub>A</sub>Rs than for example TPA023 (Atack et al, 2006) or NS 11394 (Mirza et al, 2008), and has better pharmacokinetic properties in mice than L-838,417 (Scott-Stevens et al, 2005). Before we analyzed its antihyperalgesic actions in GABA<sub>A</sub>-mutated mice, we had to verify that the H101R point mutation abolished modulation by HZ166. Although it has previously been shown that this mutation dramatically reduces the binding of and facilitation by diazepam (Wieland et al, 1992), it can have different effects on the action of other benzodiazepine site ligands. The potentiating effect of bretazenil for example is enhanced in the point-mutated receptors, and the action of Ro 45-1513 is converted from negative to positive modulation (Benson

et al, 1998). We therefore compared the binding affinity of HZ166 with wild-type  $\alpha 2/\beta 3/\gamma 2$  and point-mutated  $\alpha 2(\text{H101R})/\beta 3/\gamma 2$ -GABA<sub>A</sub>Rs (Figure 2a). In wild-type  $\alpha 2/\beta 3/\gamma 2$ -GABA<sub>A</sub>Rs, HZ166 displaced [<sup>3</sup>H] Ro 45-1513 binding with a  $K_i$  of  $221 \pm 22$  nM (mean  $\pm$  SEM,  $n = 4$ ). In  $\alpha 2(\text{H101R})/\beta 3/\gamma 2$  point-mutated receptors competition was abolished at HZ166 concentration  $\leq 10$   $\mu\text{M}$ . Only at 100  $\mu\text{M}$ , a small displacement was detected ( $26 \pm 3\%$ , mean  $\pm$  SEM,  $n = 4$ ). Subsequent electrophysiological experiments in HEK293 cells transfected with either wild-type or point-mutated  $\alpha 2(\text{H101R})/\beta 3/\gamma 2$  GABA<sub>A</sub>Rs verified that both receptors responded similarly to GABA (Figure 2b). We then investigated the impact of the point mutation on potentiation of  $\alpha 2/\beta 3/\gamma 2$  GABA<sub>A</sub>Rs by HZ166 and showed that HZ166 completely lost its ability for positive allosteric modulation in the point-mutated receptors (Figure 2c–e). Although HZ166 potentiated GABA-evoked currents through wild-type receptors by  $232 \pm 20\%$  (HZ166, 5  $\mu\text{M}$ ,  $n = 8$ ), potentiation was virtually absent in the point-mutated receptors at HZ166 concentrations  $\leq 1$   $\mu\text{M}$  and reached only  $17.6 \pm 2.4\%$  at 5  $\mu\text{M}$  ( $n = 7$ ;  $P < 0.001$  unpaired student *t*-test, wild-type vs point-mutated receptors).

Before proceeding to behavioral experiments, we also investigated the impact of *hoxb8*-cre-mediated ablation of GABA<sub>A</sub>  $\alpha 2$  subunits on the amplitude of native GABA<sub>A</sub>Rs in the spinal dorsal horn and on their sensitivity to HZ166 (Figure 3). To this end, we used lumbar spinal cord slices and recorded GABAergic membrane currents from neurons located in the superficial dorsal horn of *hoxb8- $\alpha 2^{-/-}$*  mice and of wild-type ( $\alpha 2^{\text{fl/fl}}$ ) littermates. Superfusion



**Figure 2** HZ166 binding properties to recombinant wild-type and  $\alpha 2(\text{H101R})\beta 3\gamma 2$ -GABA<sub>A</sub>Rs and potentiation by HZ166. (a) Binding affinity of HZ166 to wild-type and  $\alpha 2(\text{H101R})\beta 3\gamma 2$  point-mutated GABA<sub>A</sub>Rs determined by [<sup>3</sup>H] Ro15-4513 displacement.  $K_i$  in wild-type GABA<sub>A</sub>Rs was  $221 \pm 22$  nM (mean  $\pm$  SEM,  $n = 4$ ). (b–e) Electrophysiological analyses. (b) Activation of wild-type and  $\alpha 2(\text{H101R})\beta 3\gamma 2$  GABA<sub>A</sub>Rs exhibited similar dependence on GABA concentration.  $\text{EC}_{50}$  was  $27 \pm 1$   $\mu\text{M}$  (mean  $\pm$  SEM,  $n = 8$ ) and  $40 \pm 2$   $\mu\text{M}$  ( $n = 7$ ) for wild-type and  $\alpha 2(\text{H101R})\beta 3\gamma 2$  point-mutated GABA<sub>A</sub>Rs, respectively. Hill coefficients were  $1.2 \pm 0.03$  and  $1.4 \pm 0.07$ , and maximum currents were  $3.6 \pm 0.4$  nA and  $3.5 \pm 0.7$  nA. (c–e) Potentiation by HZ166. (c) Example of current traces evoked by GABA ( $\text{EC}_{50} = 5$   $\mu\text{M}$ ) in the presence or absence of HZ166 (1  $\mu\text{M}$ ). (d) Concentration–response curves of wild-type and  $\alpha 2(\text{H101R})\beta 3\gamma 2$  point-mutated GABA<sub>A</sub>Rs for HZ166.  $\text{EC}_{50}$  of HZ166 for wild-type GABA<sub>A</sub>Rs:  $217 \pm 20$  nM. (e) Statistical comparison of potentiation by HZ166 (1  $\mu\text{M}$ ). \*\*\* $P < 0.001$  (wild-type vs point-mutated receptors), unpaired Student's *t*-test.



**Figure 3** Effects of HZ166 on GABA-evoked membrane currents in *hoxb8-α2<sup>-/-</sup>* mice and in wild-type ( $\alpha 2^{fl/fl}$ ) littermates. (a) Whole-cell voltage-clamp recordings of GABA (50  $\mu$ M)-evoked membrane currents in superficial dorsal horn neurons of wild-type ( $\alpha 2^{fl/fl}$ ) (top) and *hoxb8-α2<sup>-/-</sup>* mice (bottom). HZ166 (10  $\mu$ M) was coapplied together with GABA. Bicuculline (bic; 20  $\mu$ M) was added at the end of the experiment to verify that all currents measured were GABA<sub>A</sub>R currents. (b, c) Statistical analysis of GABA<sub>A</sub>R current amplitudes (b) and of their potentiation by 10  $\mu$ M HZ166 (c).  $n = 6$  and 8 cells for wild-type ( $\alpha 2^{fl/fl}$ ) and *hoxb8-α2<sup>-/-</sup>* mice, respectively. \* $P < 0.05$ , unpaired Student's *t*-test.

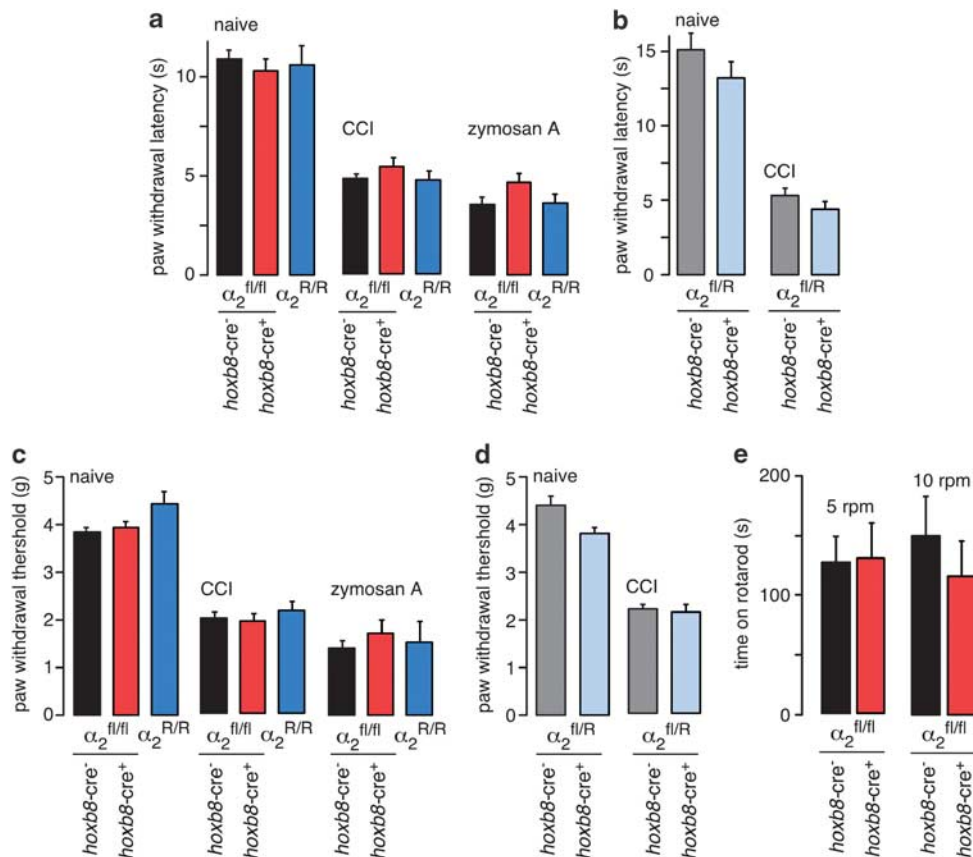
of the slices with 50  $\mu$ M GABA elicited average currents of  $306 \pm 73$  pA (mean  $\pm$  SEM,  $n = 6$ ) in wild-type mice and smaller currents in neurons of *hoxb8-α2<sup>-/-</sup>* mice ( $153 \pm 20$  pA,  $n = 8$ ,  $P = 0.04$ , unpaired student *t*-test) (Figure 3a,b). HZ166 (10  $\mu$ M) potentiated GABA<sub>A</sub>R currents in both genotypes. However, potentiation was significantly smaller in *hoxb8-α2<sup>-/-</sup>* mice ( $52 \pm 14\%$ ,  $n = 8$ ) than in wild-type ( $\alpha 2^{fl/fl}$ ) littermates ( $151 \pm 39\%$ ,  $n = 6$ ,  $P = 0.02$ , unpaired student *t*-test) (Figure 3c). Amplitudes of GABA<sub>A</sub>R currents were thus reduced by about half, whereas potentiation by HZ166 was reduced by two-thirds suggesting the presence of both benzodiazepine (HZ166)-sensitive and benzodiazepine (HZ166)-insensitive GABA<sub>A</sub>R current components in spinal dorsal horn neurons. The retention of benzodiazepine-sensitive GABA<sub>A</sub>R currents in *hoxb8-α2<sup>-/-</sup>* mice is in line with the results of our qRT-PCR experiments, which had demonstrated the expression of three benzodiazepine-sensitive GABA<sub>A</sub>R  $\alpha$ -subunits ( $\alpha 1$ ,  $\alpha 3$ , and  $\alpha 5$ ) in addition to  $\alpha 2$  (Figure 1c). Conversely, unchanged expression of  $\rho$ -subunits likely explains the reduced benzodiazepine (HZ166) sensitivity of GABA<sub>A</sub>R currents in *hoxb8-α2<sup>-/-</sup>* mice.

We then went on to investigate *hoxb8-α2<sup>-/-</sup>* and *hoxb8-α2<sup>R/-</sup>* mice in behavioral pain models and addressed their susceptibility to antihyperalgesia by systemically applied HZ166. The analysis of *hoxb8-α2<sup>R/-</sup>* mice was particularly interesting here because the unchanged expression of  $\alpha 2$ -GABA<sub>A</sub>Rs in the spinal cord (compare Figure 1b) rendered compensatory and adaptive changes highly unlikely. In addition to *hoxb8-α2<sup>-/-</sup>* mice and *hoxb8-α2<sup>R/-</sup>* mice (and their *hoxb8-cre*-negative littermates), we also included homozygous point-mutated mice ( $\alpha 2^{R/R}$ ). The comparison of HZ166-induced antihyperalgesia in  $\alpha 2^{R/R}$  mice and in wild-type mice allowed us to determine the total contribution of  $\alpha 2$ -GABA<sub>A</sub>Rs to antihyperalgesia by HZ166, independent of their location. Conditional *hoxb8-α2<sup>-/-</sup>* knock-out mice and conditional *hoxb8-α2<sup>R/-</sup>* knock-in mice as well as the *hoxb8-cre*-negative ( $\alpha 2^{fl/fl}$  and  $\alpha 2^{R/fl}$ ) littermates and global  $\alpha 2^{R/R}$  point-mutated mice responded

similarly to mechanical stimulation of their hindpaws with electronic von Frey filaments and to thermal stimulation with defined radiant heat (Figure 4a–d). Neuropathic pain sensitization induced through chronic constriction injury (CCI) of the sciatic nerve, and inflammatory hyperalgesia evoked by local subcutaneous zymosan A injection also developed similarly in all genotypes (Figure 4a–d). As a further control experiment, we assessed motor coordination in *hoxb8-α2<sup>-/-</sup>* and wild-type ( $\alpha 2^{fl/fl}$ ) littermates in the rotarod test at two different fixed rotational speeds (5 and 10 r.p.m.). Both lines managed to remain on the rotarod for similar time periods (Figure 4e).

Finally, we addressed the antihyperalgesic action of HZ166 against neuropathic pain in the three types of mutant mice (*hoxb8-α2<sup>-/-</sup>*, *hoxb8-α2<sup>R/-</sup>* and  $\alpha 2^{R/R}$  mice) and in the *hoxb8-cre*-negative wild-type ( $\alpha 2^{fl/fl}$ ) and  $\alpha 2^{R/fl}$  littermates. For these experiments, we chose a dose of 16 mg/kg body weight applied intraperitoneally (i.p.), which has previously been shown to produce a saturating antihyperalgesic response in the absence of confounding sedation or motor impairment (Di Lio et al, 2011). We first verified that this dose of HZ166 had no effect on motor coordination or locomotor activity in neuropathic mice (Figure 5a–c). We then tested the effects of HZ166 against mechanical and thermal hyperalgesia in neuropathic mice (Figure 5d–g). The antihyperalgesic drug response reached a maximum between 60 and 90 min after drug injection (Figure 5d and f). This time interval was subsequently used to quantify HZ166-induced antihyperalgesia, expressed as percent maximum possible effect (MPE). In wild-type (*hoxb8-cre*-negative  $\alpha 2^{fl/fl}$ ) mice, HZ166 reduced thermal and mechanical hyperalgesia by  $59.9 \pm 1.4\%$  and  $61.5 \pm 1.5\%$  of pre-drug values (% MPE, mean  $\pm$  SEM,  $n = 6$ , each) (Figure 5e and g). Both the time course and the degree of antihyperalgesia were very close to those reported previously by our group for wild-type C57BL/6J mice (Di Lio et al, 2011). Antihyperalgesia by HZ166 was reduced in  $\alpha 2^{R/R}$  mice to  $24.7 \pm 3.6\%$  and  $26.6 \pm 7.0\%$  of the maximum possible effect (thermal and mechanical hyperalgesia,





**Figure 4** Characterization of baseline nociceptive sensitivity and of neuropathic and inflammatory hyperalgesia and of baseline motor coordination. (a) Baseline heat sensitivity, and neuropathic (CCI) and inflammatory (zymosan A) heat hyperalgesia in  $hoxb8-\alpha_2^{-/-}$  mice ( $n = 13, 6,$  and  $7$ , for naïve, CCI, zymosan A-injected mice), in wild-type ( $\alpha_2^{fl/fl}$ ) littermates ( $n = 27, 12,$  and  $15$ ), and in separately bred global  $\alpha_2^{R/R}$  point-mutated mice of the same genetic background ( $n = 13, 6,$  and  $7$ ).  $P > 0.25$ ; ANOVA followed by Bonferroni *post hoc* test. (b) Same as a, but spinal cord-specific  $\alpha_2$ (H101R) point-mutated ( $hoxb8-\alpha_2^{R/R}$ ) mice ( $n = 6$ , for both naïve and CCI mice) and  $hoxb8$ -cre-negative  $hoxb8-\alpha_2^{R/R}$  littermates ( $n = 7$ , for both naïve and CCI mice).  $P > 0.26$ , unpaired student *t*-test. (c, d) Same as a, b, but mechanical sensitivity and hyperalgesia.  $P > 0.51$ , ANOVA followed by Bonferroni *post hoc* test (c), and  $P > 0.9$  unpaired Student's *t*-test (d).  $n = 6-7$  mice, for all genotypes, pain models and tests. (e) Motor coordination assessed in the rotarod test. Time (mean  $\pm$  SEM) for which the mouse managed to remain on the rod rotating at two different rotational velocities. Unpaired student's *t*-test,  $P = 0.45$ , for  $hoxb8-\alpha_2^{-/-}$  ( $n = 8$ ) and wild-type ( $\alpha_2^{fl/fl}$ ) littermates ( $n = 11$ ), respectively.

respectively,  $n = 6$ , each), indicating that about 60% of the antihyperalgesic actions of HZ166 were mediated by  $\alpha_2$ -GABA<sub>A</sub>Rs. The degrees of antihyperalgesia observed in both  $hoxb8-\alpha_2^{-/-}$  mice ( $24.0 \pm 5.1\%$  and  $25.0 \pm 5.1\%$ ,  $n = 6$  each, for thermal and mechanical hyperalgesia, respectively) and  $hoxb8-\alpha_2^{R/R}$  mice ( $27.4 \pm 5.1\%$  and  $26.4 \pm 5.2\%$ ,  $n = 6$  each) were virtually indistinguishable from that in global  $\alpha_2^{R/R}$  point-mutated mice, indicating that supraspinal  $\alpha_2$ -GABA<sub>A</sub>Rs were not required for antihyperalgesia by systemic HZ166. We also investigated antihyperalgesic properties of HZ166 against thermal inflammatory hyperalgesia (Figure 5h). In wild-type ( $hoxb8$ -cre-negative  $\alpha_2^{fl/fl}$ ) mice, HZ166 reduced thermal hyperalgesia by  $58.7 \pm 11.5\%$  ( $n = 7$ ). This antihyperalgesia was almost lost in  $hoxb8-\alpha_2^{-/-}$  mice ( $9.7 \pm 4.7\%$ ,  $n = 7$ ) and in global  $\alpha_2$ -point-mutated ( $\alpha_2^{R/R}$ ) mice ( $3.8 \pm 0.2\%$ ,  $n = 7$ ). Antihyperalgesia against mechanical stimuli was relatively small in wild-type mice ( $26.1 \pm 9.4\%$ ,  $n = 7$ ) and almost completely lost in  $hoxb8-\alpha_2^{-/-}$  mice ( $4.7 \pm 12.5\%$ ,  $n = 8$ ). However, the difference between wild-type and  $hoxb8-\alpha_2^{-/-}$  mice did not reach statistical significance ( $P = 0.19$ ) and effects of

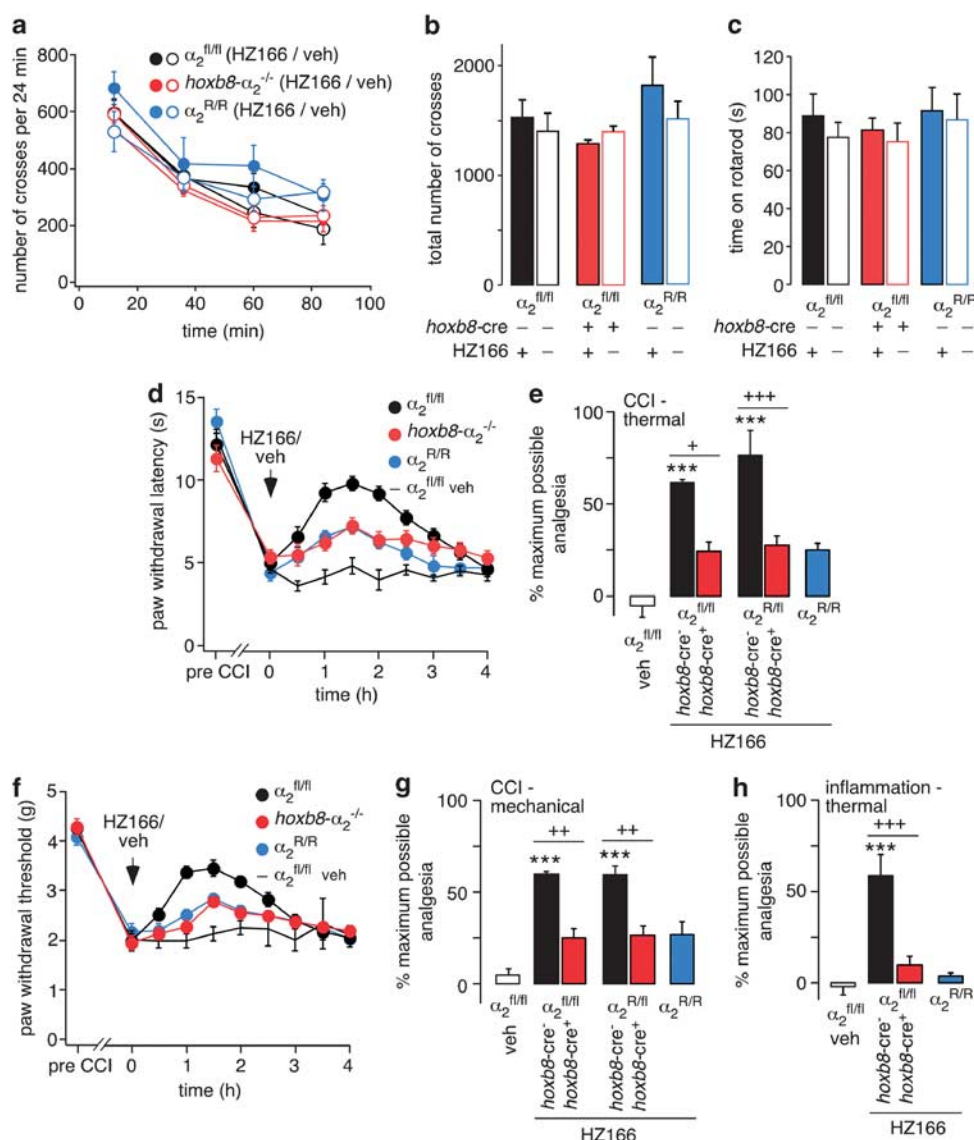
conditional deletion or mutation of the  $\alpha_2$  subunit were therefore not further investigated.

The results of our experiments not only exclude that analgesia or antihyperalgesia by HZ166 requires supraspinal  $\alpha_2$ -GABA<sub>A</sub>Rs, but also render significant indirect effects (such as reversal of anxiety or stress induced hyperalgesia) through supraspinal  $\alpha_2$ -GABA<sub>A</sub>Rs unlikely. They thus provide firm evidence for a genuine antihyperalgesic action of systemically applied benzodiazepine site agonists through a specific interaction with nociceptive circuits at the spinal level.

## DISCUSSION

Studies performed in mice carrying point-mutated benzodiazepine-insensitive GABA<sub>A</sub>Rs have allowed us to attribute the different *in vivo* actions of benzodiazepines to distinct GABA<sub>A</sub> subtypes (Möhler *et al*, 2002). In the course of these studies, it was shown that the sedative actions of benzodiazepines depend on  $\alpha 1$ -GABA<sub>A</sub>Rs, (McKernan *et al*,





**Figure 5** Locomotor activity, motor coordination and antihyperalgesia by HZ166 in  $\alpha_2$ -GABA<sub>A</sub> receptor-mutant mice. (a, b) Effects of HZ166 (16 mg/kg body weight i.p.) on locomotor activity in  $hoxb8-\alpha_2^{-/-}$ ,  $\alpha_2^{fl/fl}$ ,  $\alpha_2^{R/R}$  mice with neuropathic hyperalgesia (7 days after CCI surgery). (a) Time course. Total number of beam crosses (mean  $\pm$  SEM) per 24 min over time (0–96 min). (b) Statistical analysis. Two-way ANOVA followed by Bonferroni *post hoc* test. Genotype treatment  $F(5,42) = 1.15$ ,  $P = 0.35$ . (c) Same genotypes and treatments as in a, b, but effects on motor coordination. Time spent on an accelerating rotarod (rotational velocity increasing from 4 r.p.m. to 40 r.p.m. within 5 min) (s, mean  $\pm$  SEM). Genotype treatment  $F(5,42) = 0.36$ ,  $P = 0.87$ . (d–g) Antihyperalgesic actions of the same dose of HZ166 in conditional knock-out ( $hoxb8-\alpha_2^{-/-}$ ) and conditional knock-in ( $hoxb8-\alpha_2^{R/R}$ ) mice with neuropathic hyperalgesia (7 days after CCI surgery). (d) Changes in heat hyperalgesia (paw withdrawal latency, s, mean  $\pm$  SEM) over time after HZ166 (16 mg/kg) or vehicle administration. (e) Statistical analysis. ANOVA followed by Bonferroni *post hoc* test.  $F(5,31) = 15.9$ . \*\*\* $P < 0.001$  significant against vehicle. + $P < 0.05$ , and +++ $P < 0.001$  significantly different from  $hoxb8$ -cre-negative littermates. (f, g) Same as d, e, but mechanical hyperalgesia (paw withdrawal thresholds, g).  $F(5,39) = 13.57$ . ++ $P < 0.01$  significant against  $hoxb8$ -cre-negative littermates. (h) Same as e, but antihyperalgesic effects of HZ166 against inflammatory heat hyperalgesia 48 hrs after zymosan A injection.  $F(3,25) = 17.4$ .  $P < 0.001$ , significant against  $hoxb8$ -cre-negative littermates; \*\*\* $P < 0.001$  significant against vehicle.

2000; Rudolph *et al*, 1999), whereas the anxiolytic actions were caused by a facilitation of  $\alpha_2$ -GABA<sub>A</sub>Rs (Löw *et al*, 2000). Other studies employing the same mouse lines helped to establish additional, less obvious potential indications for subtype-selective benzodiazepine site agonists (Rudolph and Knoflach, 2011). One example of such a previously unforeseen action is antihyperalgesia, ie, the reversal of pathologically exaggerated sensitivity to pain. Although several studies in rodents had suggested pain-

modulating actions of benzodiazepine site agonists, it has been notoriously difficult to distinguish apparent analgesia or antihyperalgesia from confounding sedation. Work with GABA<sub>A</sub>R point-mutated mice provided for the first time compelling evidence for a pain-relieving action of spinal benzodiazepines and demonstrated a critical role of  $\alpha_2$ -GABA<sub>A</sub>Rs in this process (Knabl *et al*, 2008). In these experiments, pain relief manifested primarily in a reversal of pathologically increased pain sensitivity rather than in

reduced responses to acute noxious stimuli, indicating that spinal benzodiazepines exerted a hyperalgesic rather than a genuine analgesic effect.

The availability of non-sedative benzodiazepine site agonists suitable for systemic administration prompted the question about a possible role in antihyperalgesia of  $\alpha 2$ -GABA<sub>A</sub>Rs residing in supraspinal circuits. This appears as an important issue because  $\alpha 2$ -GABA<sub>A</sub>Rs are not only found in the spinal cord but also in supraspinal CNS areas where they mediate for example the anxiolytic effects of classical benzodiazepines (Löw *et al*, 2000).  $\alpha 2$ -GABA<sub>A</sub>Rs in the brain might have contributed to benzodiazepine-induced antihyperalgesia through a genuine effect on the supraspinal nociceptive circuits, eg, in the rostral insular cortex (Jasmin *et al*, 2003). Alternatively, their antihyperalgesic effects could also have been secondary, reflecting eg, a reversal of anxiety-induced hyperalgesia (Andre *et al*, 2005; Vidal and Jacob, 1982). Furthermore, supraspinal GABA<sub>A</sub>Rs in the brainstem, in particular in the rostroventromedial medulla, are known to inhibit antinociceptive fiber tracts descending to the spinal cord and might thereby produce a pronociceptive effect (Harris and Westbrook, 1995; Luger *et al*, 1995; Tatsuo *et al*, 1999). In the present study, we have measured nociceptive withdrawal responses to address these questions. Although these nociceptive responses are primarily mediated by spinal circuits (ie, they remain in spinalized animals, see for example Schouenborg *et al* (1992)), they are highly susceptible to modulation by supraspinal pain control centers, such as the rostral insular cortex (Jasmin *et al*, 2003), the amygdala (Carrasquillo and Gereau, 2007), the rostroventromedial medulla (Tatsuo *et al*, 1999), and the periaqueductal gray (Harris and Westbrook, 1995). As such, they are well-suited for investigating the effects of supraspinal GABA<sub>A</sub> receptors on hyperalgesia. In the present study, the actions of HZ166 on nociceptive withdrawal responses were nearly identical in  $\alpha 2^{R/R}$ , *hoxb8- $\alpha 2$ <sup>-/-</sup>* and *hoxb8- $\alpha 2$ <sup>R/-</sup>* mice, indicating that supraspinal  $\alpha 2$ -GABA<sub>A</sub>Rs did not have a detectable influence on HZ166-mediated antihyperalgesia. The present data therefore unambiguously demonstrate that the major (ie, the  $\alpha 2$ -GABA<sub>A</sub>R-mediated) component of antihyperalgesia by benzodiazepines occurs through a genuine effect on the spinal cord and that this antihyperalgesia is not secondary to effects of benzodiazepines on neuronal circuits in the brain. Our results hence also disprove the possibility that a reversal of anxiety-induced or stress-induced hyperalgesia contributed significantly to the antihyperalgesia measured in our experiments.

Previous studies had used intrathecal injections of diazepam or related benzodiazepines at the lumbar spinal level to demonstrate pain-relieving actions of benzodiazepines or of GABA<sub>A</sub>R agonists (reviewed in Zeilhofer *et al*, 2009). A critical role of  $\alpha 2$ -GABA<sub>A</sub>Rs in neuronal circuits of the spinal cord was therefore likely, yet still not proven, as the compounds injected might have reached supraspinal sites through rostral diffusion. The present work establishes that the spinal cord is the most relevant site for the antihyperalgesic actions of benzodiazepine site agonists.

In a previous study, we have examined the contribution of a subset of spinal  $\alpha 2$ -GABA<sub>A</sub>Rs, which reside on the central terminals of primary nociceptive afferent fibers (Witschi *et al*, 2011). This subset of  $\alpha 2$ -GABA<sub>A</sub>Rs was specifically

ablated through *sns*-cre-mediated *gabrb2* gene deletion. The respective *sns- $\alpha 2$ <sup>-/-</sup>* mice were analyzed in the same inflammatory and neuropathic pain models that have been used in the present study. These previous experiments had revealed that primary afferent  $\alpha 2$ -GABA<sub>A</sub>Rs make a partial (about 50% of the total  $\alpha 2$  component) contribution to inflammatory antihyperalgesia. The present study shows that the  $\alpha 2$ -GABA<sub>A</sub>R-mediated component of inflammatory antihyperalgesia was completely lost in *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice and hence entirely of spinal origin. Analysis of the *sns- $\alpha 2$ <sup>-/-</sup>* mice in neuropathic pain models had revealed that  $\alpha 2$ -GABA<sub>A</sub>Rs on primary nociceptors did not make any contribution to neuropathic antihyperalgesia. Again this antihyperalgesic action was completely lost in *hoxb8- $\alpha 2$ <sup>-/-</sup>* mice and therefore also exclusively of spinal origin. Early *in situ* hybridization studies had found no  $\alpha 2$ -GABA<sub>A</sub>Rs on intrinsic dorsal horn neurons (Persohn *et al*, 1991; Wisden *et al*, 1991), but more recent work provided clear evidence for the expression of these receptors by excitatory and inhibitory neurons in the spinal dorsal horn (Paul *et al*, 2012), which is in line with the data presented here.

Subsequent to the discovery that  $\alpha 2$ -GABA<sub>A</sub>Rs are the major target for the anxiolytic actions of benzodiazepines, a significant number of benzodiazepine site agonists have been developed which show reduced sedative properties through improved  $\alpha 2$  over  $\alpha 1$  subtype selectivity (Rudolph and Knoflach, 2011). These compounds allowed an assessment of the potential analgesic and antihyperalgesic actions of such compounds after systemic administration in wild-type mice without confounding sedation. Studies testing these newly developed compounds revealed significant analgesic or antihyperalgesic properties in rodent pain models (Di Lio *et al*, 2011; Knabl *et al*, 2008; Nickolls *et al*, 2011, for a review see Zeilhofer *et al*, 2012). Comparison of the antihyperalgesic efficacies of different compounds with their pharmacological profiles at different GABA<sub>A</sub>R subtypes suggests that a rather high intrinsic activity at  $\alpha 2$ -GABA<sub>A</sub>Rs and a high  $\alpha 2$  over  $\alpha 1$  selectivity profile are necessary for significant antihyperalgesia in the absence of sedation (Zeilhofer *et al*, 2012). Although these results were consistent with the findings obtained in the GABA<sub>A</sub>R point-mutated mice discussed above, final proof that these antihyperalgesic effects indeed originated from  $\alpha 2$ -GABA<sub>A</sub>Rs was missing. Here we focused on one such compound, the novel partial benzodiazepine site agonist HZ166. The present study demonstrates that the antihyperalgesic actions of HZ166 were to a large extent mediated by  $\alpha 2$ -GABA<sub>A</sub>Rs (about 90% and 60% for inflammatory and neuropathic hyperalgesia, respectively).

Antihyperalgesia was not completely lost in the different GABA<sub>A</sub>R  $\alpha 2$ -mutant mice investigated here. Depending on the model used (ie, inflammatory or neuropathic hyperalgesia), between 10 and 40% of the total antihyperalgesia were retained in *hoxb8- $\alpha 2$ <sup>-/-</sup>*, *hoxb8- $\alpha 2$ <sup>R/-</sup>*, and  $\alpha 2^{R/R}$  mice. This is consistent with our previous study employing intrathecal diazepam injections, where between 30 and 50% of the antihyperalgesia remained in  $\alpha 2^{R/R}$  mice. At the spinal level, this remaining component was mediated by  $\alpha 3$ -GABA<sub>A</sub>Rs and/or  $\alpha 5$ -GABA<sub>A</sub>Rs (Knabl *et al*, 2008). It is likely that these spinal receptors also account for the antihyperalgesia retained in HZ166-treated *hoxb8- $\alpha 2$ <sup>-/-</sup>* and *hoxb8- $\alpha 2$ <sup>R/-</sup>* mice. For a given benzodiazepine site

agonist, the actual contribution of  $\alpha 2$ -GABA<sub>A</sub>R vs  $\alpha 3$ -GABA<sub>A</sub>Rs and  $\alpha 5$ -GABA<sub>A</sub>Rs will depend on its potentiating effects at these GABA<sub>A</sub>R subtypes. Until similar studies as the present one have also been performed for  $\alpha 3$ - and  $\alpha 5$ -GABA<sub>A</sub>Rs, it cannot be excluded that GABA<sub>A</sub>Rs different from  $\alpha 2$  (ie,  $\alpha 3$ -GABA<sub>A</sub>Rs and  $\alpha 5$ -GABA<sub>A</sub>Rs) also contribute through a supraspinal site.

The present study provides strong evidence for a genuine antihyperalgesic action of systemically applied non-sedative benzodiazepine site agonists and demonstrates the pivotal contribution of spinal cord circuits to this antihyperalgesia. A critical role of inhibitory neurons and neurotransmitter receptors in the spinal dorsal horn has been first proposed in the gate control theory of pain (Melzack and Wall, 1965), but attempts to translate this concept to pain therapy have largely been unsuccessful. The present results show that an enhancement of fast GABAergic inhibition in the spinal dorsal horn is a possible strategy to reverse pathological hyperalgesia. Provided that the results obtained with genetic mouse mutants translate to the action of  $\alpha 2$ -GABA<sub>A</sub>R selective drugs in humans, these agents should be devoid of sedation and memory impairment (Rudolph and Knoflach, 2011). Conversely, it is likely that such drugs will exert in addition to their antihyperalgesic actions also anxiolytic and possibly muscle relaxant properties (Rudolph and Knoflach, 2011), both of which should be beneficial to many chronic pain patients. The present finding therefore provide additional impetus for the development of subtype-selective benzodiazepine site agonists as novel antihyperalgesic agents.

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